A method to maintain mammalian cells for days alive at 4 °C

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Abstract

This paper describes a method for the temporary storage of cultured cells. Cells from recently completed cell monolayers were trypsinized and then centrifuged. After centrifugation, the supernatant and pellet were kept at 4 °C for one week. After storage, the supernatant was discarded, the cells were resuspended and used for seeding new flasks and for titration of virus. The cells not only remained viable, but also rapidly formed new monolayers and allowed immediate infection and growth of viruses. We conclude that this method can be a helpful asset to cell culture experiments.

Introduction

Establishing the growth kinetics of adherent monolayers of cells is not feasible without destroying the monolayers. In contrast with liquid cultures of bacteria, estimations of cell counts by optical density are not possible, and, consequently, precise predictions of when a cell culture will reach a desired density cannot be made. As a consequence, working with animal cell cultures requires pre-planning in order to have cells of appropriate density and physiological conditions needed for the experimental design. But, frequently, a good planning does not ensure having the cells ready for use when programmed.

The growth of cells in culture usually follows a standard pattern. When cell density reaches saturation of the surface available for attachment, or when the cell concentration exceeds the capacity of the medium, growth ceases or is greatly reduced. Then either the medium must be changed more frequently, or the culture must be divided or pre-

served. The preservation of cell cultures has been usually achieved by reducing the fetal serum concentration (holding media) and the temperature from 37 to 30 °C, to maintain cell lines with a finite lifespan without using up the limited number of cell generation available to them. In non-proliferating cultures, cells still metabolize some constituents of the medium become exhausted or degrade spontaneously (Freshney 2000). After cell preservation in holding medium, it is recommended to subculture cells before their use in biological assays because the cell properties typical of the exponential phase of cell growth may have changed. This procedure is cumbersome and costly.

Deep-freezing may be the only method for long-term (from months to years) preservation of cells. However, in some cases, short-term (72–96 h) storage methods may be sufficient. For example, a temperature of around 4 °C is recommended for hypothermic liquid storage, in order to avoid intracellular ice crystal formation caused by

freezing, and, after sedimentation, blood cells can be kept at 4 °C for relatively long periods of time (up to 3 weeks). In addition, peripheral blood stem cells can be preserved at 4 °C or at subzero non-freezing temperatures (Matsumoto et al. 2002) for up to 72 h. These cells maintain their functionality; consequently, it is assumed that most of their metabolic activities remain intact.

On the basis of these precedents, here we developed a new method for the storage of cells at 4 °C for up to 3–4 days. With this approach, cells can be used directly after storage for the following purposes: the rapid formation of a monolayer, and virus infection and replication in methods that involve the infection of suspended cells, such as the suspended cell plaque assay (Copper 1961; Dahling and Wright 1988), the VIRADEN method (Papageorgiou et al. 2000) and the double-layer plaque assay (Mocé-Llivina et al. 2004).

Materials

1. Cell lines

- a. BGM cell line (European Collection of Animal Cell Cultures, accession no. 90092601).
- b. CaCo2 cell line (ATCC HTB37)

2. Viruses

- a. Poliovirus 1 vaccine strain Sabin 1 Lsc 2ab
- b. Echovirus 6 (environmental isolate)
- c. Echovirus 25 (environmental isolate)
- d. Coxsackievirus B5 (environmental isolate)

3. Cell culture media and reagents

- a. Eagle's MEM (11-100-24, ICN Biomedicals) containing 5% fetal bovine serum (F2442, Sigma), 26.8 mM sodium bicarbonate (S 8761, Sigma), Hepes buffer (BE17-737E, BioWhittaker), 2 mM L-glutamine (BE17-605E, BioWhittaker), 100 units ml⁻¹ streptomycin, 100 µg ml⁻¹ penicillin (DE17-602E, BioWhittaker) for the culture of BGM cells.
- b. Eagle's MEM containing 10% fetal bovine serum, 26.8 mM sodium bicarbonate, Hepes buffer, non-essential aminoacids, 2 mM L-glutamine, 100 units ml⁻¹ streptomycin, 100 µg ml⁻¹ penicillin for the culture of CaCo2 cells.

- c. Medium for preparing cell suspension: E-MEM containing 1% FBS, 26.8 mM sodium bicarbonate, Hepes buffer, non-essential aminoacids, 2 mM L-glutamine, 100 units ml⁻¹ streptomycin, 100 µg ml⁻¹ penicillin, 50 µg ml⁻¹ gentamicin (G1272, Sigma), 50 units ml⁻¹ nystatin (N1638, Sigma), and 20 µg ml⁻¹ ceftazidime (Fortum, GlaxoSmithKline).
- d. Trypsin–EDTA solution: 0.25% of Trypsin 1:250 (Difco) and 0.02% of EDTA in PBS pH 7.1.

Procedures

Preparation of cell suspension and storage conditions

We cultured BGM cells for 3–4 days and CaCo2 cells for 6–7 days. Cell suspensions were prepared as follows. Cell monolayers were grown on 175 cm² flasks and were then washed twice in PBS pH 7.1 and trypsinized using 5 ml of trypsin–EDTA solution. When cells started to detach, they were diluted into fresh suspension medium (described above) to obtain suspensions containing from 2×10^6 to 5×10^6 cells per ml. These suspensions were centrifuged at $3000 \times g$ for 10 min. After centrifugation, the tubes with the pellet fraction and the entire remaining suspension medium were stored at 4 °C without pellet resuspension.

To recover the cells after storage, the supernatant was discarded and the cells were resuspended in 1 ml of suspension medium. For both BGM and CaCo2 cells, the cell concentration following this procedure ranged from 2×10^7 to 5×10^7 cells per ml.

Cell counts and cell viability

This test was performed only with BGM cells stored at 30 and at 4 °C. After storage, cell counts were manually estimated using a hemocytometer. Trypan blue dye exclusion was used to estimate the percent viability of cells (Freshney 2000). Viable cells were counted daily from day 0 to day 8.

Cell functionality

At the end of the storage period, the growth capacity and virus titration capacity of BGM and CaCo2 cells were tested.

Growth capacity assays

These were used to evaluate the effect of the storage at 4 °C on the growth capacity of BGM and CaCo2 cells. After a range of storage periods (0–8 days), the cells were resuspended and seeded on 25 cm² flasks with 2×10^5 cells per cm². At the same time, for controls, cells obtained from recently completed monolayers were subcultured and seeded in control flasks with the same cell concentration as above. The growth of the cells to achieve confluent monolayers was compared with that of control cells.

Virus titration capacity assays

To examine the effect of the storage method on the capacity of viral infection, several enterovirus suspensions were enumerated in BGM and CaCo2 cells stored at 4 °C and in cells that had not been stored. Viruses were titrated using the VIRADEN method (Papageorgiou et al. 2000) and the double layer plaque assay (Mocé-Llivina et al. 2004). Both methods require a confluent monolayer and cell suspension. Cells stored at 4 °C were used directly as cell suspension for virus enumeration with these methods. Several enteroviruses were titrated: vaccine strain of poliovirus 1 (Sabin 1, Lsc 2ab), echovirus 6, echovirus 25 and coxsackievirus B5. Viral suspensions were obtained previously by replication on BGM cells (Freshney 2000).

Data processing and analysis

The results of viral determinations were applied to the so-called Shewhart control charts constructed to follow a quality control of the viral suspensions maintained at –70 °C and used for the quantification methods (Anonymous 1991). These charts show whether the quality of analysis performed on distinct days or in distinct conditions is ‘in control’. However, a Shewhart control chart can also show whether and when the viral counts change significantly – numbers above or below the upper (average plus 3 σ) and lower (average minus 3 σ) control limits – with respect to the batch.

Safety considerations

All cultures were assumed to be hazardous and the following safety precautions were taken:

- a biological safety cabinet was used;
- pipette aids were used to prevent ingestion and the use of aerosols was kept to a minimum;
- work surfaces were disinfected (before and after procedure).

Results and discussion

After storage at 4 °C, the viability of the BGM cells was maintained for over 97.9% of the total numbers of cells from day 0 to 8 (Table 1). In contrast, cells stored at 30 °C with maintenance medium, showed a constant loss of total numbers in the monolayer, which reached around 75% after 8 days. Most detached cells suspended in maintenance medium were not viable according to trypan blue dye exclusion. The decrease in the viability of the cells remaining in the monolayer, 94.3%, was similar to that of those stored at 4 °C. The data illustrate that the viability of cells after a storage period of 4 °C was between 4 and 5 times higher than that of those in the monolayers of the cultures kept in maintenance medium at 30 °C.

The cell functionality assays showed that cells maintained growth capacity and viral infection capacity up to 4 days storage at 4 °C. In growth capacity assays, the flasks used to culture cells were examined daily, comparing each condition with its corresponding control. Cells stored at 4 °C for 48 h presented the same degree of viability and growth capacity to achieve confluence as the recently subcultured control cells (Table 2).

For virus titration capacity assays, three independent experiments were performed. Control charts were done previously for each virus assayed (Figure 1) in order to establish when the titers of the viruses obtained with cells stored at 4 °C differed significantly from controls. In all cases, virus counts were consistent with controls when titration was performed with cells stored at 4 °C for 96 h (Table 3). An exception was observed only in the titration of echovirus 6 with BGM cells. The reason for this difference is unknown. This virus may have a receptor in this cell line that is inactivated more quickly than the others, but evidence in support of this hypothesis is not available. Our results show that the cells stored under the conditions described here have sufficient viability to be used for viral testing with methods that require cells in suspension,

Table 1. Viability of BGM cells stored in MEM 1% FBS at 30 °C in culture flasks vs. viability of BGM cells centrifuged and stored at 4 °C.

Time(days)	Cell stored at 30 °C		Cells stored at 4 °C	
	Viability (%) of cells attached in the monolayer	Number of viable cells in the monolayer (cells cm ⁻²)	Viability (%)	Number of viable cells (cells cm ⁻²) in the pellet
0	100 (0.0)	2.6×10^4	100 (0.0)	2.6×10^4
1	97.8 (0.9)	7.7×10^3	99.7 (0.4)	2.3×10^4
2	98.4 (0.5)	9.2×10^3	99.3 (0.4)	2.8×10^4
3	96.8 (0.4)	9.5×10^3	98.5 (0.7)	2.6×10^4
6	97.6 (0.9)	6.5×10^3	97.82 (1.4)	2.7×10^4
7	95.4 (1.1)	7.5×10^3	97.7 (0.9)	2.9×10^4
8	94.3 (1.1)	6.5×10^3	97.9 (0.6)	2.9×10^4

Table 2. Effect on monolayer formation (growth capacity) of cells stored at 4 °C.

Cell line	Time at 4 °C (h)	Control seems	Cell growth at 37 °C after seeding			Suspended cells in the medium ^a
			At 24 h	At 48–72 h	At 4–5 days	
BGM	24	Identical	++	+++	+++	—
	48	Identical	++	+++	+++	—
	72	Similar	++	+++	+++	+
	96	Similar	+	++	+++	+
	192	Similar	+	++	+++	+
CaCo2	24	Identical	++	++	+++	—
	48	Identical	++	++	+++	—
	72	Similar	+	++	+++	+
	96	Similar	+	++	+++	+

+++ : confluent growth; ++ : more or less confluent growth; + : adhered cells and started growth.

^a— : absence of suspended cells in growth medium; + : presence of suspended cells in growth medium.

Figure 1. Poliovirus 1 (Sabin) counted on BGM cells stored at 4 °C (◆). Control chart (□) performed previously. Broken lines are the upper (average plus 3σ) and the lower (average minus 3σ) limits of the control chart.

Table 3. Titration capacity of enteroviruses with BGM cells and CaCo2 cells stored at 4 °C.

Time (h)	BGM cells				CaCo2 cells			
	Polio 1 (Sabin)	Echo 6	Echo 25	Coxsackie B5	Polio 1 (Sabin)	Echo 6	Echo 25	Coxsackie B5
0	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+
48	+	+	+	+	+	+	+	+
72	+	+	+	+	+	+	+	+
96	+	—	+	+	+	+	+	+
192	—	—	—	nt	nt	nt	nt	nt

Enumeration of enteroviruses obtained with cells stored at 4 °C were compared with control enumerations performed previously using control charts.

+: pfu enumerations inside the upper and lower action limits of the control chart; —: pfu enumerations below the lower action limit of the control chart.

The results were the same for VIRADEN and the double-layer plaque assay.

nt: not tested.

such as the suspended cell plaque assay (Copper 1961; Dahling and Wright 1988), the VIRADEN method (Papageorgiou et al. 2000) and the double-layer plaque assay (Mocé-Llivina et al. 2004).

After sedimentation and resuspension, peripheral blood stem cells stored for several days at 4 °C show a viability around 100% (Matsumoto et al. 2002). This finding is consistent with our results on BGM cells. However, we observed that BGM cells kept at 4 °C maintained intact capacity to rapidly grow on monolayers and to support viral infection and replication after at least 72 h storage. In contrast, Matsumoto et al. (2002) showed that peripheral blood stem cells lost more than 20% of their capacity to establish colony forming units after 24 h storage.

Although we applied our method in the context of virological research work, we consider that it may be useful those working with cell cultures, since it saves time, expensive material and may help to increase the lifespan of cell lines.

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